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FINAL REPORT

PRESERVATION OF BIOLOGICAL MATERIALS BY FREEZE-DRYING

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ABSTRACT

The purpose of this study was to investigate the feasibility of freeze-dehydrating biological materials by extracting frozen moisture with dry cryogenic liquids in the presence of dessicants. Freeze-dehydration of hydrated gelatin capsules in liquid nitrogen and liquid nitrous oxide proceeded too slowly to be evaluated. Dehydration was feasible in ethyl ether and ethyl alcohol at dry ice-alcohol temperature (-78.5°C). The presence of a dessicant in the dehydration system was found to be essential for complete drying. A sequential vacuum freeze drying-nitrous oxide critical point dehydration technique was used to obtain porous dehydrated meat tissue. Dehydration of partially freeze dried meat tissue (10% residual moisture) was also carried to completion ($< 1\%$ moisture) in a dry liquid at -78.5°C in the presence of molecular sieves. Preliminary data indicated that microorganisms can be similarly dehydrated with retention of viability.

Preservation of Biological Materials by Freeze-Drying

I. Introduction

This final report covers research conducted for the Office of the Surgeon General on contract No. DA-49-193-MD-2297, entitled, "Preservation of Biological Materials by Freeze-Drying". The period of the report is from July 1, 1962 to March 31, 1964.

The purpose of the study was to investigate the feasibility of dehydrating biological materials by extracting frozen moisture with superdry liquids. Work reported in the literature by Mueller and Szent-Gyorgi[1] had shown that freeze-drying of muscle in acetone at dry ice temperature does not denature protein and is capable of extracting water. In their technique the frozen muscle was kept in acetone for 3 weeks in order to obtain a dehydrated sample. Apparently, molecular structure and contractility were not destroyed during the storage period. The proposed technique entailed circulating superdry cryogenic liquids around frozen biological specimens. Moisture extracted by the liquids would in turn be removed by passage of the liquid through molecular sieves or other suitable drying agents. The dry liquids would then be recirculated again as a dehydrating medium.

The rate of freeze-drying under vacuum is governed by the rate of diffusion of water molecules. Despite the fact that diffusion in liquids is slower than in gases, it was thought that the rate of water removal would be more rapid when frozen moisture is extracted with liquids for the following reasons:

1. The mechanical sweeping action of densely packed molecules in a liquid is much greater than that obtained with the rarefied packing of molecules in gases.

2. Heat of melting is lower than heat of sublimation.

3. The rate of heat transfer is faster in liquids than in gases and very considerably faster than in vacuum.

4. Liquids like alcohol, acetone, etc. show a great physicochemical affinity for water. This effect is employed to advantage in rapid moisture determinations with Karl Fischer reagents. This method is considerably faster than the conventional moisture procedures of drying in air or even in vacuum.

5. Contact of liquid with the specimen is much better (molecules are at closer distance from each other) than in a vacuum (or even gas).

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1. Mueller, H. and A. Szent-Gyorgi, Science 126 970-1, 1957.

Consequently, the total mass of extracted moisture per unit of time should be greater in a liquid, though the specific diffusion rate may be slower.

This report describes the various research approaches investigated to endeavor to dehydrate biological specimens in cryogenic liquids. Critical temperature dehydration, vacuum freeze-drying followed by critical temperature dehydration, and dehydration in superdry cryogenic liquids, were also investigated during the present study.

II. Experimental Studies

A thorough literature search was conducted as the initial phase of work. Information was compiled on: (1) effects of freezing parameters on the structure and viability of biological materials, (2) properties of dessicants, (3) characteristics of liquid nitrogen and liquid nitrous oxide freezing systems, and (4) evaluation of freeze-dried materials.

In the initial experimental studies the unit illustrated in Fig. 1 was used to determine whether superdry liquids will extract moisture from frozen hydrated gelatin capsules. Gelatin was selected as the substrate because it is a protein which can be uniformly hydrated to desired moisture levels. Gelatin capsules (15 x 5 x 0.1 mm) were cut into a flower petal shape, placed in a copper wire screen basket, and submerged in water at refrigeration temperature for varied time intervals. Capsules weighing approximately 0.04 g gained approximately 0.3 g water in 3 hrs and 0.4 to 0.5 g overnite. The baskets containing the hydrated gelatin capsules were placed in glass tubes (25 x 300 mm) containing a dessicant and the extracting liquid added thereafter. Dry ice-alcohol (-78.5°C) and liquid nitrogen (-196°C) were used as cooling baths.

Molecular sieves and magnesium perchlorate were the dessicants employed to absorb water which might diffuse through the extracting liquid from the frozen gelatin. The dessicants also maintained the liquid in a super dry state during freeze-drying. Karl Fischer moisture determinations were made on the liquid and gelatin residues after freeze-drying. The molecular sieves were regenerated by heating in a muffle furnace at 600 to 650°C with an air dryer blowing warm air over the surface of the sieves. Molecular sieves containing 1.5% water dry in 15 min., those containing 5 to 7 % dried in 3 hrs.

The water of hydration of the gelatin capsules is quantitatively removed by drying in an air oven at 105°C for 2 hrs. Thus, by adding the extracted water to that obtained from drying the gelatin residue in the oven, and dividing by the initial water of hydration one can determine the percent water recovery. A comparison of the weight of the dried residue to the original capsule weight will also indicate whether gelatin has been lost in handling.

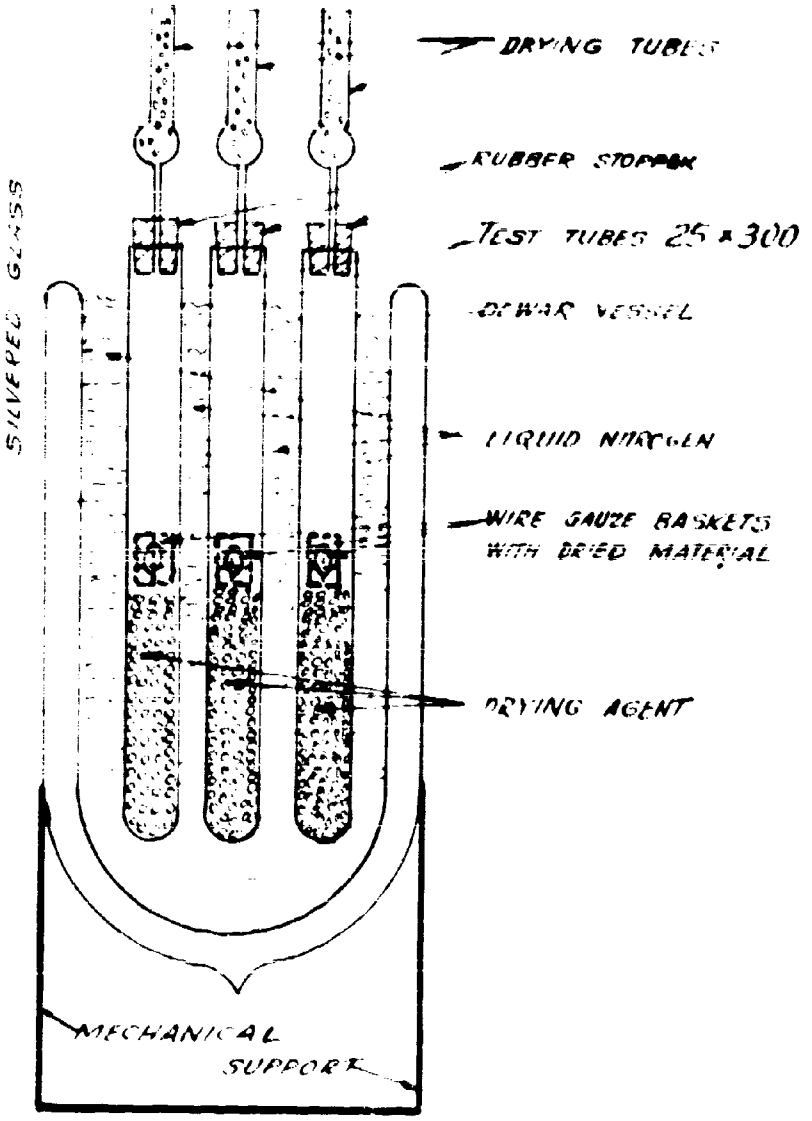
DRYING TUBES
RUBBER STOPPER
TEST TUBES 25 x 300
DEWAR VESSEL
LIQUID NITROGEN
WIRE GAUZE BASKETS WITH DRIED MATERIAL
DRYING AGENT
SILVERED GLASS
MECHANICAL SUPPORT

FIG. 1

A. Freeze Dehydration at Dry Ice-Alcohol Temperature (-78.5°C)

1. Absolute Alcohol and Ethyl Ether

Experimental trials were conducted to determine the percent moisture removed from hydrated gelatin capsules during storage in absolute alcohol and ethyl ether at dry ice temperature. Hydrated gelatin capsules were placed in a precooled glass tube containing 50 g molecular sieves and to this was added 50 ml of the liquid medium. As shown in Table 1, moisture is extracted from hydrated gelatin at dry ice temperatures by absolute alcohol and ethyl ether in the presence of a dessicant.

Table 1
Freeze-Drying of Hydrated Gelatin in Absolute Alcohol and Ethyl Ether at Dry Ice-Alcohol Temperature

Sample No.	Liquid Medium	Water of Hydration g	Time in Liquid plus molecular sieves hrs	Moisture Loss g	Extracted Water of Hydration %
1	Absolute Alcohol	0.502	24	0.462	92
2	Absolute Alcohol	0.376	48	0.372	99
3[a]	Absolute Alcohol	0.368	72	0.319	87
4	Ethyl Ether	0.404	48	0.387	96
5[b]	Ethyl Ether	0.555	72	0.510	92

[a] Molecular sieves were not predried

[b] Used gelatin previously freeze-dried in absolute alcohol (sample 2) and rehydrated

2. Petroleum Ether (boiling range 30-60°C)

A series of experiments were performed to determine: (1) whether moisture from a frozen gelatin capsule will diffuse through an immiscible liquid; (2) whether the presence of a dessicant in the liquid facilitates moisture removal; and (3) the effectiveness of molecular sieves versus magnesium perchlorate as a dessicant. A summary of the results obtained in these exploratory tests is presented in Table 2.

Table 2
Freeze-Drying of Hydrated Gelatin in Petroleum Ether at Dry Ice-Alcohol Temperature

Sample No.	Water of Hydration g	Dessicant	Time in Liquid hrs	Moisture Loss	Extracted Water of Hydration %
6	0.432	-	96	0.067	15.5
7	0.388	-	96	0.053	13.7
8	0.476	-	96	0.125	26.2
9	0.343	Mol.sieves	24	0.025	7.3
10	0.295	Mol.sieves	24	0.041	13.9
11	0.290	Mol.sieves	48	0.060	20.6
12	0.284	Mol.sieves	48	0.049	17.3
13	0.291	Mol.sieves	96	0.083	28.6
14	0.501	Mg. perchlorate	24	0.128	25.6
15	0.499	Mg. perchlorate	48	0.366	73.5
16	0.435	Mg. perchlorate	96	0.346	79.5
17	0.636	Mg. perchlorate	96	0.318	50.0

The results indicate that water does diffuse through an immiscible liquid, that magnesium perchlorate is a more effective dessicant than molecular sieves, and that the presence of a dessicant in the liquid medium accelerates moisture extraction.

B. Freeze-Dehydration at Liquid Nitrogen Temperature (-196°C)

Freezing and drying in liquid nitrogen is of special interest since the structure and viability of a number of biological materials can only be preserved at this temperature during extended storage. The series of experiments shown in Table 3 were performed to obtain information on moisture extraction at liquid nitrogen temperature. These preliminary results indicated that moisture is slowly extracted from a frozen biological material in a static system at liquid nitrogen temperature.

The experimental units illustrated in Figs. 2 and 3 were designed to endeavor to increase the rate of water extraction from frozen gelatin capsules by continuously circulating a super dry liquid across the surface of the frozen specimen.

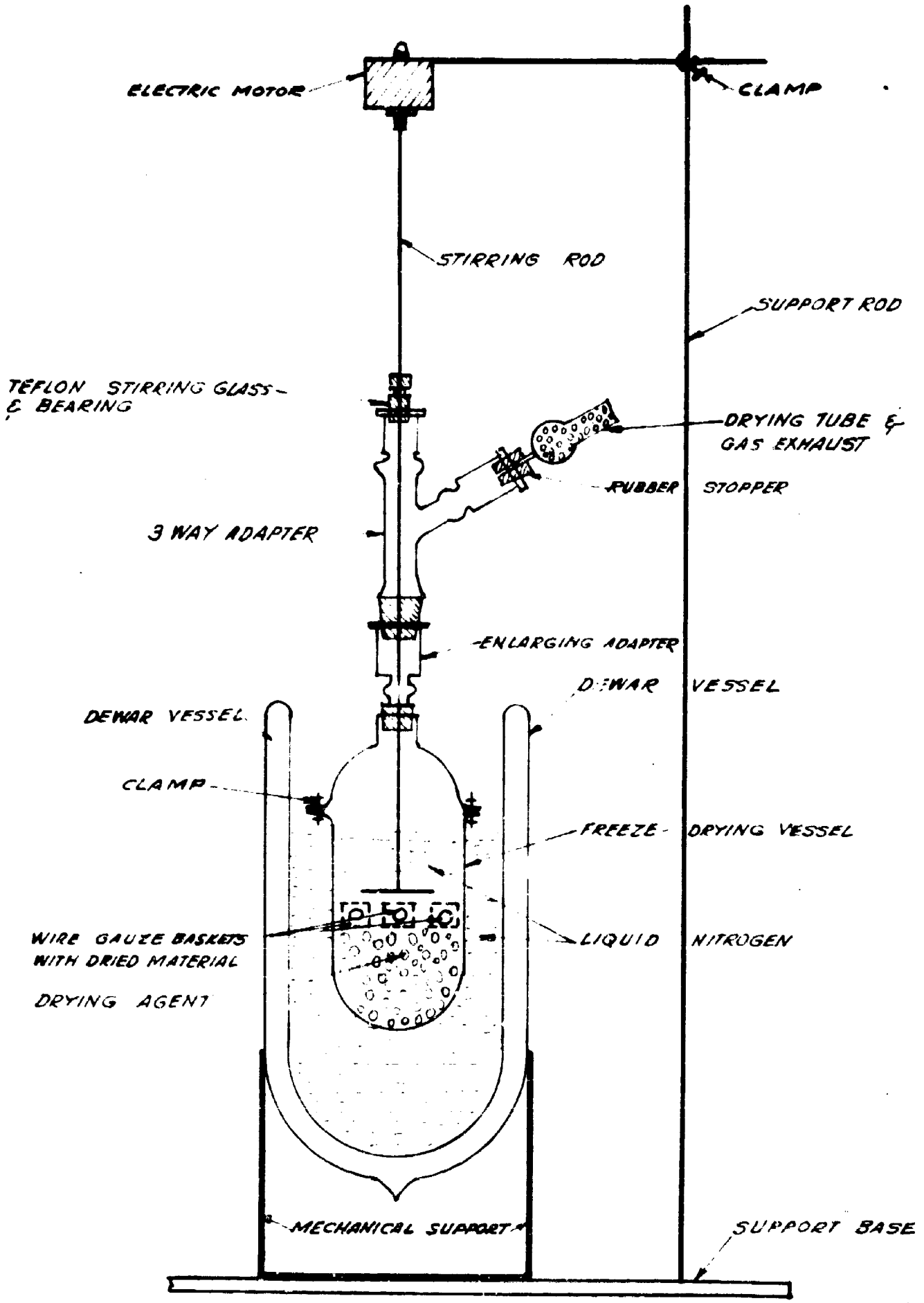
ELECTRIC MOTOR
CLAMP
STIRRING ROD
SUPPORT ROD
TEFLON STIRRING GLASS & BEARING
DRYING TUBE & GAS EXHAUST
RUBBER STOPPER
3 WAY ADAPTER
ENLARGING ADAPTER
DEWAR VESSEL
DEWAR VESSEL
CLAMP
FREEZE-DRYING VESSEL
WIRE GAUZE BASKETS WITH DRIED MATERIAL
DRYING AGENT
LIQUID NITROGEN
MECHANICAL SUPPORT
SUPPORT BASE

FIG. 2

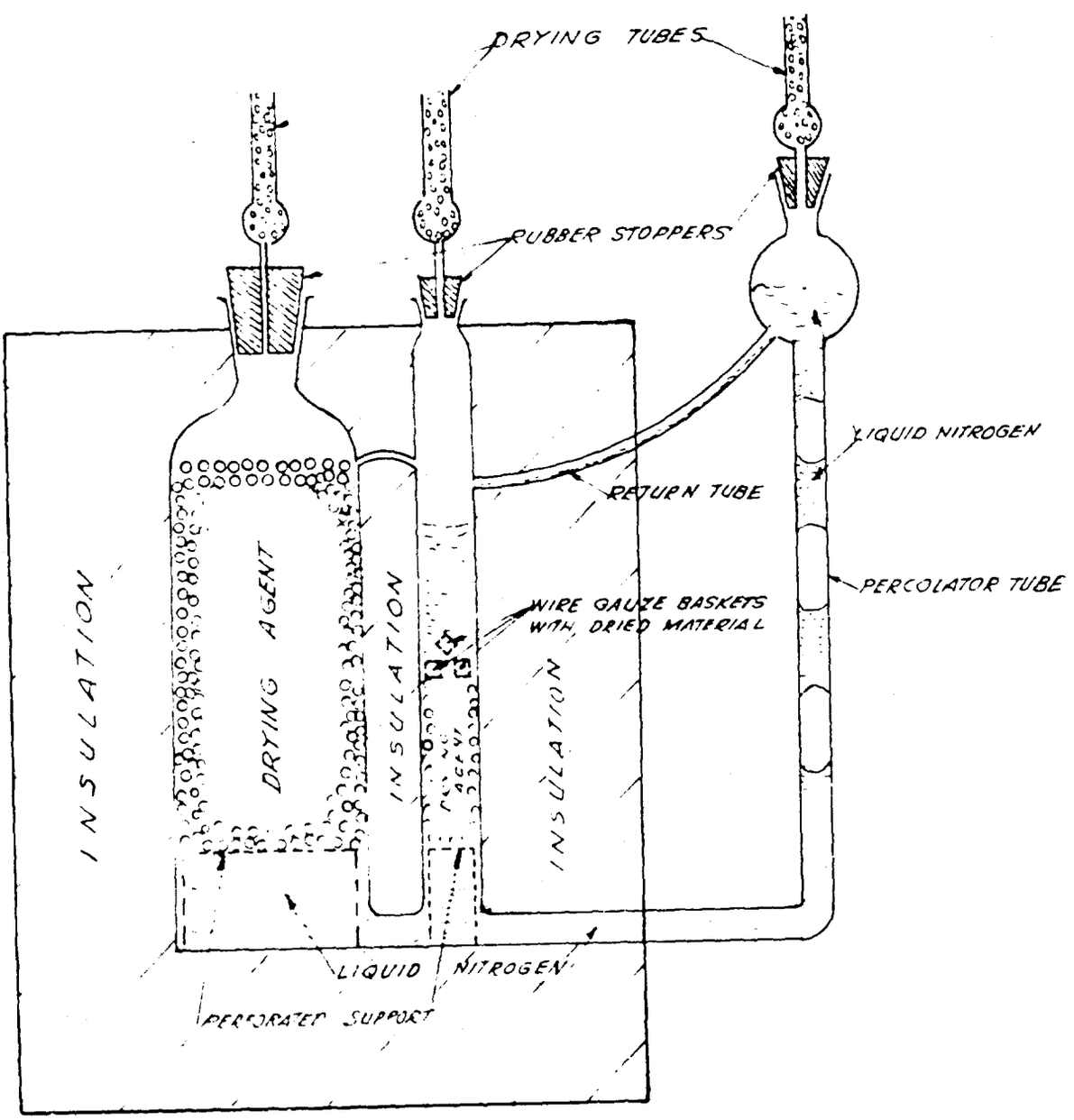
DRYING TUBES
RUBBER STOPPERS
LIQUID NITROGEN
RETURN TUBE
PERCOLATOR TUBE
WIRE GAUZE BASKETS WITH DRIED MATERIAL
INSULATION
DRYING AGENT
INSULATION
INSULATION
LIQUID NITROGEN
PERFORATED SUPPORT

FIG. 3

Table 3
Freeze-Drying of Hydrated Gelatin with Liquid Nitrogen in Liquid Nitrogen Bath

Sample No.	Water of Hydration g	Dessicant	Time in Liquid hrs	Moisture Loss g	Extracted Water of Hydration %
18	0.318	-	96	0.014	4.4
19	0.289	-	96	0.026	9.0
20	0.475	Mol. sieves	96	0.094	19.8
21	0.305	Mol. sieves	96	0.081	26.6
22	0.315	Mg. perchlorate	48	0.131	41.5
23	0.272	Mg. perchlorate	120	0.122	45.0
24	0.273	Mg. perchlorate	200	0.156	57.0

The procedure employed with the system illustrated in Fig. 2 was as follows:

> Hydrated gelatin capsules in individual wire mesh baskets were placed in a one liter Virtis freeze-drying flask containing approximately 250 g dessicant. The flask was lowered into a 4 liter Dewar containing liquid nitrogen. Previously dried liquid nitrogen was then pumped into the flask until the baskets were covered. A drying tube was inserted into the side arm of the flask and the stirrer turned on. The level of liquid nitrogen was kept constant throughout the experiment. Upon completion of the trial the flask was raised out of the Dewar, and the capsules were removed and placed in a dessicator for 5 minutes prior to weighing.

A series of experimental trials was conducted to determine moisture loss when hydrated gelatin capsules are stirred for 5 hours with liquid nitrogen and various dessicants. Previous results in tube experiments had indicated that some dessicants are more effective than others at liquid nitrogen temperature. The results of these experiments are illustrated in Table 4. Controls were run by placing hydrated gelatin capsules on top of the dessicant in the freeze-drying flask, freezing with liquid nitrogen removing the capsules from the flask without stirring, and then weighing. The data indicates that moisture was extracted in series 1-4, however, the variation in moisture loss obtained under supposedly comparable conditions of drying precludes positive conclusions. Some of the factors that conceivably could affect the results are: (1) surface area of the capsules, (2) efficiency of regenerated dessicants at liquid nitrogen

Table 4

Freeze-Drying of Hydrated Gelatin Capsules in Liquid Nitrogen

		Experimental Trials					
		[a]		[b]		[c]	
Series	Dessicant	Water of Hydration, mg	Moisture loss, mg	Water of Hydration, mg	Moisture loss, mg	Water of Hydration, mg	Moisture loss, mg
1	Molecular Sieves Type 13x	339	110	330	82		
		290	109	368	109		
		317	144	365	99		
		324	118	345	53		
		326	96	400	85		
		292	73				
	Avg.	315	108	362	86	None	None
2	Molecular Sieves Type 10x	417	130	343	68		
		387	101	368	103		
		432	135	357	79		
		469	129	369	81		
		446	85	401	98		
				359	66		
	Avg.	430	116	366	83	None	None
3	Microtraps Type Z-14	417	115	339	97	425	116
		403	117	394	114	349	72
		401	120	362	97	370	134
		374	110	257	96	377	154
		395	80	244	115		
				254	122		
	Avg.	398	108	308	107	380	119

a. Capsules stirred for 5 hrs in liquid nitrogen containing dessicants (250g.)

Table 4 (continued)

Freeze-Drying of Hydrated Gelatin Capsules in Liquid Nitrogen

Series	Dessicant	Experimental Trials					
		Water of Hydration, mg	Moisture Loss, mg	Water of Hydration, mg	Moisture Loss, mg	Water of Hydration, mg	Moisture Loss, mg
4	Magnesium perchlorate on vermiculite	472	101	405	132	473	127
		399	102	389	155	396	89
		351	89	442	176	381	80
		385	92	397	153	425	94
		427	150	411	169	405	73
		373	70	418	62	401	139
	Avg.	401	101	410	141	414	100
5	Magnesium perchlorate 12 mesh	420	77				
		436	75				
		439	66				
		450	81				
	Avg.	436	75				
6	Magnesium perchlorate (G.F.Smith Co.)	346	45				
		356	40				
		369	53				
		338	67				
		380	40				
		379	63				
	Avg.	361	51				
7	Activated Alumina	436	85				
		408	83				
		455	84				
		469	82				
	Avg.	442	84				

8 Control -- Average of 20 determinations; moisture loss = 54 mg. (range 45 to 80 mg)

(3) location of the capsules in the flask with respect to the circulation pattern of the stirrer and proximity to the dessicant bed, (4) moisture loss during handling of the frozen capsules, and (5) porosity of the capsule surface.

Material balance studies were undertaken to endeavor to differentiate actual moisture loss due to freeze-dehydration from "apparent" moisture loss due to handling and other experimental variables. It was determined that 50 to 70 mg of moisture evaporated between the time the hydrated capsules were weighed and frozen in the drying flask; approximately 8 to 10 mg were lost when the partially dehydrated capsules were removed from the flask for reweighing. Obviously, extreme care must be taken to prevent evaporation of moisture from hydrated samples, and moisture condensation or evaporation from frozen samples.

C. Dehydration with Liquid Nitrous Oxide (-88.5°C)

Liquid nitrous oxide theoretically binds 6 molecules of water and forms a hydrate. To determine whether this strong affinity for water could be applied to the drying of frozen hydrated gelatin capsules, a series of experiments were performed using liquid nitrous oxide obtained from the Liquid Carbonic Company, Chicago.

In the procedure, gelatin capsules were hydrated, weighed, and immediately frozen by immersion into liquid nitrogen. The frozen capsules were then placed in a freeze-drying flask containing 250 g Davison microtraps 542. The flask was submerged into a Dewar vessel containing liquid nitrous oxide, and then filled with nitrous oxide poured in from another Dewar vessel. An electric stirrer was used to stir the nitrous oxide during the experimental trial. Upon completion of the trial, the capsules were removed from flask, placed in a dessicator for 2 minutes, and weighed. Moisture mist condensed on the surface of the capsules appeared to be removed in the dessicator. Control capsules were similarly treated except that they were removed from the flask after 5 minutes in the nitrous oxide bath. Results obtained are shown in Table 5. It is apparent from the table that moisture removal is slow under the conditions of the experiment.

Table 5
Freeze-Drying of Hydrated Gelatin Capsules in Liquid Nitrous Oxide[a]

Control Samples		Samples	
Water of Hydration mg	Moisture Loss mg	Water of Hydration mg	Moisture Loss mg
361	5	360	55
316	11	350	57
307	3	365	50
334	5	373	33
356	10	353	49
360	2	351	25
avg. 339	6	359	45

[a] capsules stirred for 24 hrs in liquid nitrous oxide containing microtraps (250g)

D. Acceleration of Moisture Removal at Cryogenic Temperatures

The slow rates of dehydration obtained in liquid nitrogen (-193°C) and liquid nitrous oxide (-88.5°C) indicated that more rapid moisture removal would be necessary to obtain completely dehydrated samples. A number of experimental approaches were evaluated for this purpose.

1. Effect of mutual solubility properties of liquids on moisture removal

Tinmermans[2] notes that adding a substance which is soluble in only one or two liquids in a mixture causes a lowering of the solubility of both liquids in each other. However, if the added substance is miscible with both liquids then its presence will increase the solubility of both liquids in each other.

Liquid ammonia and Freons were considered as additives to liquid nitrogen - hydrated gelatin systems in the presence of molecular sieves. It was thought that either of these substances might possibly act as a solubility bridge by removing water from the gelatin and having it absorbed in the dessicant. Experiments performed revealed that ammonia is not miscible with liquid nitrogen, forming whitish flakes when dispersed in the liquid nitrogen. Freons 12 and 13 are miscible with liquid nitrogen, however, they have a very limited solubility for water (approximately 0.1 ppm at -100°F). Freon 21 has a greater solubility for water (12 ppm at -100°F), but is insoluble at liquid nitrogen temperature. Preliminary experiments conducted with Freon 21 - absolute alcohol systems at dry ice-alcohol temperature did not show enhanced moisture removal. Further work on this approach was placed in abeyance until a substance is uncovered which has a greater affinity for water and is more miscible with liquid nitrogen or nitrous oxide than is presently available.

2. Application of heat during dehydration

With conventional vacuum freeze-drying and liquid extraction, it is thought that the rate of freeze-drying is not limited by mass transfer (i.e. passage of water vapor from the interior) but rather by heat transfer. Accordingly, it appeared likely that increased rates of moisture removal at cryogenic temperatures might be obtained by applying external heat to the specimen during dehydration.

Briskeat fiber glass insulated heating tapes[3] were placed inside test tubes and the temperatures calibrated by rheostat settings. In the procedure the tubes were coated on the outside with a 10% gelatin solution, weighed, and immersed into the cryogenic liquids. The current transmitted through the tapes during dehydration approximated 0.8 amperes. Although 10% moisture loss was

2. Tinmermans, J. Zeitschrift fur Physikalische Chemie 58 129-213, 1907
3. Brisco Manufacturing Company, Columbus, Ohio

achieved in 3 to 5 hrs as compared with 12 hrs without heating, further significant moisture removal was not obtained. Apparently the surface of the gelatin capsules dehydrates rapidly and further diffusion of moisture into the cryogenic liquids becomes limited. Additional tests confirmed the initial observations that the extent of moisture removal from frozen gelatin at cryogenic temperatures was not enhanced by the levels of current employed for these studies.

3. Measurement of moisture loss at cryogenic temperatures

Considerable difficulty was experienced in obtaining reproducible and accurate measurements of moisture removed from hydrated gelatin at liquid nitrogen and liquid nitrous oxide temperatures. Due to the wide temperature range between liquid nitrogen (-193°C) and ambient temperature (23°C), moisture condenses immediately on the surface of the capsules as they are removed from the liquid nitrogen in the Dewar vessel. Although controls were run concurrently to compensate for this effect, the question arose whether such an indirect procedure accurately measures moisture extracted.

To obviate the effect of moisture condensing on the cold gelatin surfaces, radioactive tritiated water was used for hydrating the gelatin capsules prior to immersion in liquid nitrogen or liquid nitrous oxide. Radioactivity was measured in microcuries per milligram of sample before and after treatment. A Tri-Carb liquid scintillation counter was used to make the radioactivity counts. The moisture loss in capsules stirred for 24 hrs in liquid nitrous oxide containing molecular sieves as a dessicant was determined to be approximately 10% by direct weighing and by tritiated water counts. This correlation suggested that weighings were sufficiently accurate for exploratory studies.

E. Drying in Absolute Alcohol and Ethyl Ether

Experiments were performed to determine whether moisture is extracted from hydrated gelatin in the presence of dessicants as readily in ether as in absolute alcohol. Considering the limited solubility of water in ether as compared to alcohol, greater solubility in ether could be indicative of a higher diffusion rate rather than an overall solubility relationship. In the experimental studies, hydrated gelatin capsules were placed in glass tubes containing 30 grams molecular sieves 3A, and 40 ml ether or alcohol. The samples were kept at room and dry ice temperatures. The data obtained is shown in Table 6. These preliminary results suggest that with hydrated gelatin capsules containing approximately 400 mg of water of hydration, moisture is extracted to a greater degree in ethyl ether than in absolute alcohol.

Table 6

Drying of Hydrated Gelatin Capsules in Absolute Alcohol and Ethyl Ether

Solvent	Time of Drying Days	Moisture Loss, % Room Temperature(23°C) without dessicant	Room Temperature(23°C) with dessicant	Dry Ice (78.5°C) without dessicant	Dry Ice (78.5°C) with dessicant
Absolute Alcohol	5	76.5	81.0	24.0	29.0
	15	77.4	79.4	27.2	32.4
Ethyl Ether	5	98.0	99.5	28.2	38.8
	15	98.4	99.5	37.3	71.4

It is apparent from the data that ethyl ether extracts more moisture than does absolute alcohol from hydrated gelatin at both room and dry ice temperatures. The presence of a dessicant in the dehydrating solvent enhances the dehydration particularly at the dry ice temperature. These observations again emphasize that the solubility of water in the solvent may not be the basic consideration in dehydration with solvents. Ethyl ether has a limited solubility for water yet it extracted moisture more readily than ethyl alcohol which is completely miscible with water.

Another series of experiments were performed to determine whether prefreezing of hydrated gelatin capsules in liquid nitrogen might be advantageous for subsequent dehydration in ethyl ether. Prefreezing conceivably could help eliminate artifacts during freeze-drying and might also be necessary for retention of viability of frozen biological tissues. Table 7 shows that prefreezing does not adversely affect the extent of dehydration. Further work is indicated to determine whether viability is affected by prefreezing with liquid nitrogen.

Experiments were conducted to compare the dehydration of gelatin capsules in ethyl ether, petroleum ether, and absolute ethyl alcohol at dry ice and room temperatures.

Hydrated gelatin capsules were weighed and then prefrozen in either liquid nitrogen or cold ethyl ether (-78.5°C). The capsules were then placed in tubes containing 30 g Linde molecular sieves No. 5A and approximately 60 ml of cold solvent was added. The tubes were kept in a Dewar flask containing dry ice and alcohol (-78.5°C). At various time intervals, the capsules were removed from the tubes, and weighed after solvent removal by evaporation. The frozen capsules were then returned to the tubes. Controls consisted of

Table 7

Prefreezing and Drying[a] of Frozen Hydrated Gelatin Capsules

Treatment of Hydrated Capsules	Water of Hydration, mg	Water loss, mg		% Moisture Loss
frozen, drying in ethyl ether, without dessicant	76	49		64.0
	79	46		58.2
	97	55		55.3
			avg	59.2
frozen, drying in ethyl ether with dessicant	63	44		69.8
	115	43		37.2
	98	54		55.0
			avg	54.0
frozen in liquid nitrogen, drying in ethyl ether without dessicant	87	47		54.0
	87	40		45.9
	105	41		39.0
			avg	46.3
frozen in liquid nitrogen, drying in ethyl ether with dessicant	82	54		65.6
	88	57		64.7
	84	55		63.5
			avg	64.6

[a]drying at dry ice temperatures for 15 days; dessicant was molecular sieves

hydrated gelatin capsules in solvents at room temperature. Results shown in Table 8 are expressed as percentage water removal based on water of hydration (avg. of 3 detns.).

Table 8

Dehydration of Frozen Gelatin Capsules in Solvents at Dry Ice and Room Temperature

Solvent	Temperature °C	Moisture Removal, % 3 days	6 days	Total Moisture Removed, %
petroleum ether	25	80.5	19.6	100.1
	-78.5	14.0	0.0	14.0
ethyl ether	25	100.0	0.0	100.0
	-78.5	48.5	31.6	80.1
Absolute ethyl alcohol	25	80.5	9.3	89.8
	-78.5	18.5	40.5	59.0

The results again confirm previous observations that ethyl ether extracts more moisture from frozen hydrated gelatin capsules at dry ice temperature than does absolute ethyl alcohol or petroleum ether. It is of interest that in the static system described moisture diffuses from frozen capsules into both polar and non-polar solvents.

To determine whether the presence of a dessicant and stirring of the solvent enhances moisture removal, experiments were performed wherein frozen hydrated gelatin capsules were placed directly over molecular sieves in a stirring flask containing cold ethyl ether. The flask was kept submerged in a dry ice-alcohol bath (-78.5°C) throughout the run. Control samples were similarly run except that dessicant was not present in the stirring flask. The results obtained are shown in Table 9.

Table 9 Dehydration of Frozen Gelatin Capsules in an Agitating Solvent System (ethyl ether) (Linde Molecular Sieves No. 5A)

	Moisture Removal, (% of Water of Hydration).%		
Sample	13 hrs	26 hrs	39 hrs
with dessicant	44.3	63.5	66.6
without dessicant	33.9	52.7	58.5

(Each value represents an average of 6 determinations)

For purposes of comparison, drying of frozen capsules with ethyl ether in a static system (Table 8) for 3 days (72hrs.) only removed 48.5% moisture. The results shown in Table 9 indicate that stirring and the presence of a dessicant accelerates moisture removal. In approximately one day (26 hrs.), 63.5% moisture was removed from the frozen gelatin capsule in an agitating system containing dessicant.

Another variable affecting the rate and extent of moisture removal appears to be the degree of water of hydration of the capsule. As shown in Table 10, less moisture, percentage-wise, was removed per unit time from capsules with high water of hydration than from those with less hydration. Available surface area and the possibility of surface constriction are factors that could affect moisture removal rate.

Although dehydration of frozen biological materials in liquid nitrogen and liquid nitrous oxide has been found to proceed too slowly for practical purposes, dehydration in solvents at dry ice temperature (-78.5) is feasible. The solvent system must be selected on the basis that it does not adversely affect structure and viability. (Fluoroalcohols, freons, are possible solvents.)

Table 10. Effect of Degree of Water of Hydration on Moisture Extraction from Frozen Gelatin Capsules
(stirring in ethyl ether at -65° C, Linde Molecular Sieves No. 5A)

	Water of Hydration, mg	Moisture Extraction, % 15 hrs	26 hrs	39 hrs
	69.5	63.3	69.0	72.0
	83.0	59.0	67.5	67.5
	95.0	54.8	65.3	67.3
Avg.	82.5	59.0	67.3	68.8
	210.5	32.5	45.5	49.5
	204.0	36.0	45.0	49.5
	277.0-	20.8	30.3	32.1
Avg.	230.5	29.8	40.3	43.7

F. Critical Point Dehydration with Nitrous Oxide

Anderson[3] working with a carbon dioxide critical point system demonstrated that by entirely eliminating any liquid-vapour surface dehydration, the method preserves the structure of extended and fragile materials and eliminates artifacts due to surface tension. Critical point and freeze drying methods give comparable results when applied to small objects like viruses and microorganisms. However, for larger more fragile structures, i.e. red cell ghosts, dehydration is better accompliished with the critical point method, possibly because cooling and consequent brittleness of the specimen is avoided.

In the critical point method employed by Anderson, a series of miscible liquids, comprising alcohol, amyl acetate, and liquid carbon dioxide, were used as the dehydrating medium in a metal cylinder at 25° C. By raising the temperature to 45°C, (safely above the 31° C critical point of carbon dioxide) the carbon dioxide is released as a gas through an escape valve. The dehydrated sample remains in the cylinder.

3. Anderson, T.F. Proc. Intern. Conf. Electron Microscopy, London, pp 122-129, 1954.

Solvents such as alcohol, amyl acetate and liquid carbon dioxide may not adversely affect gross structure, but they could adversely affect the viability of dehydrated biological materials. Nitrous oxide is known to be innocuous and, based on our studies with dehydration in cryogenic liquids, a critical point system containing liquid nitrous oxide and molecular sieves (dessicant) appeared advantageous as a dehydration medium. Also, since dehydration of gelatin capsules in liquid nitrous oxide (-88°C) proceeds very slowly, it was of interest to determine whether liquid nitrous oxide in a critical point system would dehydrate gelatin, meat tissue and blood.

The general procedure was a modification of that described by Anderson[3] and entailed placing 75g Linde Molecular Sieves, No. 3A, in a steel cylinder (1-31/32 in. od x 12-7/8 in. long), covering the dessicant with a screen, and adding the test samples. The cylinder was sealed with a special valve[4], placed in a 4°C water bath, and connected to a nitrous oxide cylinder. Liquid nitrous oxide flows into the experimental cylinder and is allowed to stand for 30 min. to equilibrate. The large nitrous oxide cylinder is then disconnected and the small cylinder weighed to determine whether there is complete fill. Pressure in the cylinder reaches 750 lb/sq in. After a specified time interval, the cylinder is placed in a 45-47°C water bath (critical point of nitrous oxide is 36.5°C) for 20 min., whereupon the pressure rises to 1080 lb/sq in. The gas is then allowed to escape at a controlled rate to prevent cooling and reverse condensation inside of the cylinder. Moisture loss is determined by weighing, and comparisons made with oven drying values.

The moisture content of the materials used in the experimental studies were:

Material	Moisture[a] Content, % 100 (water of hydration)
gelatin	
beef tissue	71.8
ground beef	65.5
pork tissue	71.5
rabbit blood, citrated	85.5

a. Ovendrying, 15 hrs at 100°C

Initially, experiments were performed to determine the effect of time in liquid nitrous oxide and bleeding out time on the rate of dehydration and on the physical characteristics of the dehydrated materials.

4. Supplied by Liquid Carbonic Co.

As shown in Table 11, complete dehydration of blood, gelatin, and meat tissue can be obtained in a liquid nitrour oxide-dessicant critical point system. Control runs using nitrous oxide gas in place of liquid nitrous oxide under pressure, and those wherein the dessicant was removed showed significantly lower dehydration. The dehydrated materials obtained by critical point drying were not entirely satisfactory since they were not very porous and appeared shrunken. However, critical point dried specimens were more porous and less shrunken than air or dessicator dried samples.

Table 11.
Nitrous Oxide Critical Point Dehydration of Biological Materials

Material	Time in Liquid Nitrous Oxide, Hrs.	Bleeding Time, min.	Moisture Loss, %	Percent of Total Moisture (Oven Method)
Blood, rabbit, citrated	0.0	10	44.0	59.5
	2.0	14	86.5	101.0
	4.0	10	85.8	100.0
	0.0	240	87.0	102.0
	15.0	20	87.1	102.0
(Control)	15.0 --- Nitrous oxide gas, dessicant		77.2	90.4
Gelatin, hydrated	15.0	15.0	100.0	100.0
	15.0	60.0	100.0	100.0
(Control)	15.0 --- Nitrous oxide gas, dessicant		91.5	91.5
Tissue pork pieces	4.0	5 hr	43.0	60.0
	0.5	8 hr	71.5	100.0
	3.0	7 hr	73.5	103.0
	15.0	15	73.6	103.0
pork strips	0.5	10	9.5	13.3
	1.0	10	22.3	31.2
	2.0	14	33.2	46.5
	18.0	14	66.3	92.8
(Control)	15.0	Nitrous oxide gas, dessicant, no pressure	55.0	77.0
(Control)	15.0	Nitrous oxide gas, no dessicant, no pressure	10.0	14.0
(Control)	15.0	Nitrous oxide liquid, pressure, no dessicant	9.6	13.4

Table 12

Nitrous Oxide Critical Point Dehydration of Meat Tissue [1.]

Sample	Time in Liquid Nitrous Oxide hrs	Bleeding Time min	Moisture Loss %	Percent of Total Moisture (oven method)
Beef, pieces	-	-	-	100 (71.8% H_2O)
	1	10	28.1	39.2
	1	60	31.1	43.4
	1	2 (hr)	37.6	52.5
	1	4 (hr)	46.2	64.4
	2	12	26.6	37.1
	2	14	33.2	46.3
	4	10	36.2	50.5
	4	10	36.8	51.3
	4	25	36.2	50.5
	5	10	45.7	63.7
	15	10	59.6	83.1
	15	2 (hr)	63.5	88.5
	15	6 (hr)	72.5	101.0
	72	90	72.5	101.0
	72	3 (hr)	69.7	93.7
Control (minus dessicant)	15	10	9.9	13.7

Table 12. (continued)
Nitrous Oxide Critical Point Dehydration of Meat Tissue[1].

Sample	Time in Liquid Nitrous Oxide hrs	Bleeding Time min	Moisture Loss %	Percent of Total Moisture (oven method)
Beef, ground	-	-	-	100
	1	12	30.5	46.6
	1	60	29.9	45.7
	1	60	40.2	61.4
	1	2 (hr)	37.8	57.8
	1	2 (hr)	35.6	54.4
	1	2 (hr)	40.7	62.1
	1	4 (hr)	52.8	80.7
	1	4 (hr)	52.6	80.4
	1	4 (hr)	55.7	85.0
	2	10	25.0	38.2
	4	10	32.4	49.5
	5	10	35.7	54.5
	15	60	73.3	112.0
	72	3 (hr)	79.6	121.5

1. Dessicant in system was Linde Molecular Sieves No. 3A, 1/8" pellets

C. Sequential Vacuum Freeze Drying-Critical Point Dehydration

Upon further consideration, we concluded that vacuum freeze dehydration followed by critical point treatment would be an effective drying technique. Vacuum freeze-drying of meat tissue is relatively rapid down to about 10 to 15% residual moisture. Further dehydration to approximately 1% proceeds slowly since it is difficult to remove water from the moist center core. During dehydration, case hardening or contraction of the surface oftimes occurs and adversely affects reconstitution. Vacuum freeze-drying of tissue to 10 to 15% moisture (to fix the porous structure), followed by rapid completion of dehydration in a liquid nitrous oxide critical point system should permit final drying at an accelerated rate and without further alteration of structure or surface properties. This may also serve to enhance viability retention. Table 13 presents drying data obtained by the combined dehydration techniques.

The data indicates that critical point dehydration can be successfully employed to obtain complete drying following initial freeze-drying. The dehydrated tissue is porous, hydrates rapidly, and has very good surface appearance. Apparently, the critical point treatment accelerates the rate of moisture removal without significant alteration of structure. Hydration of a vacuum freeze-dried sample of ground beef approximated 82% of the original moisture content compared to 165% for a sample that was partially freeze-dried and drying completed by the critical point technique. With whole beef tissue strips, approximately 60% rehydration was obtained by both techniques. Microscopic examination of the dehydrated tissues revealed no further alteration of structure by critical point dehydration.

Examination of the data reported in Table 14 raised the question of the high moisture recoveries after critical point dehydration (up to 124%). This was particularly evident with the drying of ground meat by both the critical point dehydration and the freeze-dry critical point dehydration techniques. The high values suggested that some constituent in addition to moisture was being extracted from the meat tissue during the treatment with liquid nitrous oxide. Subsequently, we found that the difference can be attributed to extraction of lipid material by liquid nitrous oxide. With whole meat the extraction of lipids was less significant.

H. Histological Examination of Dehydrated Meat Tissues

Samples of dehydrated meat were submitted as coded numbers to Dr. Catchpole, Department of Pathology, University of Illinois Medical School, for examination of lipid composition. The actual samples were as follows:

1 - Whole pieces of beef, vacuum freeze drying and nitrous oxide critical temperature drying

2 - Whole pieces of beef, vacuum freeze drying

3 - Whole pieces of beef, vacuum freeze drying and carbon dioxide critical temperature drying

In order to prepare a solution of lipid, 40 mg of the preparations 1, 2, 3, were weighed out, finely chopped with a razor blade and extracted with 1 ml of a chloroform-methanol mixture 4:1 v/v/2 days. The extract was centrifuged but because it did not settle very well, a plug of cotton previously extracted with

Table 13

Dehydration of Meat Tissue by Vacuum Freeze Drying - Critical Point Dehydration

	FREEZE-DRYING			CRITICAL POINT DEHYDRATION			
Sample	Dry Ice-Alc. (-78.5°C) hrs	Room Temp. (25°C) hrs	Moisture Loss %	Time in Liquid Nitrous Oxide hrs	Bleeding Time min	Moisture Loss %	Percent of Original Moisture !
beef,tissue ($71.8\%\ H_2O$)	15	-	44.6	-	-	-	68.1
	15	2	53.7	-	-	-	74.8
	-	7	57.2	-	75	79.5	110.7
	3	1	44.4	15	35	74.4	103.6
	3	1	41.7	15	35	74.7	104.0
	9	2	69.4	11	60	74.7	1040
	12	-	71.3	1	120	72.9	101.4
	15	-	72.0	1	180	74.6	103.8
beef,ground ($65.5\%\ H_2O$)	-	2	55.0	1	60	80.0	122.1
	6	-	59.5	4	60	77.9	118.8
	9	2	66.0	1	60	76.8	117.2
	12	-	62.5	1	120	81.2	124.0
	15	-	59.1	15	60	78.8	120.0

chloroform-methanol was introduced into the tube and the extract could then be aspirated from solid material.

Comparison of the lipid material extracted from the three specimens was done in a semi-quantitative manner by using the method described by Hack, Biochemical J. 54, 602-5, 1953. The method provides a visual demonstration of the relative amounts and kinds of various lipids present. The method is thought to be far superior to histological diagnosis on sections. For more accurate quantitation of lipids, gas chromotography would need to be used.

Comparison 1: 0.1 ml of the solution was carefully pipetted into an 8mm pencilled circle drawn on Whatman filter paper #1. (10cm approx. circles were used.) The solution was evaporated after the application of each droplet by an air current. When all the solution had been placed, chloroform-methanol was similarly added to enlarge the spot to about 14 mm. diameter, and the spot dried by evaporation. Finally, acetone was added drop by drop so that the diameter of the wet spot increased about 2 mm per second to give a final ring of about 50 mm (this takes about half a minute). The acetone ring dries promptly and is ready for staining.

In this run it was elected to divide the circle into 90° sectors and to stain them respectively with:

alpha-	Sudan black
beta-	Periodic acid-leucofuchsin
gamma-	Oil red-O
delta-	Plasmal- Mercury bichloride-leucofuchsin.

Comparison 2: Methanol was used instead of acetone to develop the spot.

The sector in this case was divided into 5- approx. 72° sectors and stained as follows:

alpha-	Osmic acid
beta-	Plasmal
gamma-	Oil red-O
delta-	Periodic acid-leucofuchsin
epsilon-	Sudan black

In a final comparison, amounts of 0.02, 0.04, and 0.06 ml of the three solutions were pipetted into 16 mm circles and stained as a whole, after bisecting the circles with:

1. Oil red-O
2. Plasmal

These were selected as the most favorable for visual comparison. In addition, they stain essentially different groups of lipids as shown by the first two tests.

Results: Lipid was present in all three specimens. By inspection of the total lipid spots, based on oil-red-O and plasmal staining, specimen 3 may have fractionally more lipid than #2, and #2 than #1. However, only a single extract was studied, and this of a particular sampling of tissue. Thus one is more impressed with the similarities of these preparations than with their differences. Since the above two reagents pick up different classes of compounds, and since they vary, if at all, in the same sense, then these 2 classes are not differentially extracted by the original preparative agents.

In a general way, Sudan black and oil red-O stain neutral fats (triglycerides). In the acetone chromatogram these appear in the outer ring because of acetone-solubility. Here #3 looks a bit denser than #2. In the methanol chromatogram, most of these compounds do not move from point of origin. Here also #3 shows a greater density and some indication of a deeper colored halo. A small fraction moves to the periphery.

Osmium stains unsaturated fats and aldehydes which remain at the point of origin in the methanol chromatogram and were not run with acetone.

Plasmal test (Feulgen) demonstrates fatty aldehydes which were oxidised by $HgCl_2$ and then stained with leucofuchsin (Schiff's base). With acetone they remain at the origin with a slight spreading penumbra. With methanol the fraction splits with most of it going to the outer ring, some staying at the origin. In these chromatograms #2 looks more prominent than #3, but this was not conclusive.

PAS test (Periodic acid leucofuchsin). Periodic acid oxidises 1:2 glycols to aldehydes, and the compounds containing these linkages in this particular extract are no doubt glycolipids. They stay at the origin with acetone. Not much difference between 1, 2, 3. The chromatogram with methanol was poor undoubtedly due to too great dilution of the material which is largely dispersed to the periphery with this solvent. A trace of material stays at the origin.

Conclusion Extraction of preparations 1, 2, 3 with a lipid solvent showed qualitatively and semi-quantitatively the same lipid composition and amounts. There was no indication of differential extraction of lipids during the processes of preparation of the specimens.

Although lipid extraction needs to be examined further, the results obtaine do indicate that partially freeze-dried meat tissue can be completely dried by extracting residual moisture with solvent in the presence of a dessicant. By selecting a solvent system that will not extract lipids, it should be possible to develop the freeze-dry critical point dehydration procedure so that biological tissues can be dried in a relatively short time with minimal alteration of structure. Since the number of solvents available with critical point temperatures in a practical range is limited, comparable results might be obtained, albeit at a slower rate of drying, by placing partially freeze-dried samples in a cold solvent system containing dessicant. The porosity of the freeze-dried samples would permit the superdry solvent to come into contact with the residual moisture present in the core of the sample. Thus, the moisture would be extracted under mild conditions which should

not adversely affect structure or surface properties. In this manner, the elimination of artifacts during freeze-drying of biological tissue in cryogenic liquids should be feasible. Various solvents including ethyl ether, alcohol, sulfur dioxide, dichloromethane, dimethylsulfoxide, etc. could be evaluated for this purpose.

3. Viability Retention of Organisms

A series of experiments were conducted to measure the effect of liquid nitrous oxide on the viability of organisms, and to determine whether viable organisms can be dehydrated in a cold solvent containing dessicant.

1. Effect of liquid nitrous oxide at 50°C.

In the critical point dehydration procedure, the liquid nitrous oxide in the cylinder is released as a gas at a controlled rate. During the bleed time the cylinder is kept in a 50°C water bath. The question arose as to whether organisms can survive contact with liquid nitrous oxide at 50°C.

A base suspension of E. Coli in dextran (25%) containing monosodium glutamate was used as the test medium. One milliliter aliquots of the base suspension were frozen in test tubes and lyophilized. The tubes were then placed in the critical point dehydration cylinder in contact with liquefied nitrous oxide. The cylinder was immediately put into a 50°C water bath in order to reach the critical temperature, and the gas was bled over 60 and 90 min. intervals. Table 15 shows that under the conditions of the experiment approximately 70% of the organisms present after freeze-drying were inactivated. Whether this is due to the solvent action of nitrous oxide or the elevated temperature (50°C) remains to be determined. However, it is noted that 30% of the organisms retained viability under the conditions of critical point dehydration.

Table 14

Effect of Liquid Nitrous Oxide on E. Coli Suspensions

Conditions of Vacuum Freeze-Drying	Critical Temperature Bleed Time, Min.	Bacteria Count ($\times 10^4$)	Viability Retention (based on freeze-dried suspension) %
15 hrs at -65°C	-	4800	100.0
15 hrs at -65°C	60	1110	23.4
15 hrs at -65°C	60	1508	31.4
72 hrs at -65°C 2 hrs at 25°C	-	840	100.0
72 hrs at -65°C 2 hrs at 25°C	60	270	32.0
72 hrs at -65°C 12 hrs at 25°C	-	704	100.0
72 hrs at -65°C 12 hrs at 25°C	90	141	20.0

2. Viability of E. Coli in cold alcohol

Tubes containing 1 ml of bacterial suspension were frozen with dry ice and then submerged in cold alcohol (-77°C) containing molecular sieves No. 10x, 1/8" pellets. At specified time intervals individual tubes were removed from the alcohol, drained and immediately placed under vacuum at -59°C for 60 minutes to remove residual alcohol. The tubes were then diluted with sterile water and plated out. Table 15 indicates that the frozen bacterial suspensions lose viability as a function of time in contact with the cold alcohol. A more innocuous solvent system would be necessary for practical consideration of this means of dehydration.

Table 15

Effect of Alcohol and Dessicant[1.] on E. Coli

Time in Alcohol Min	Bacteria Count ($\times 10^4$)	Viability Retention %
0	1045	100.0
15	133	12.7
30	86	8.2
60	69	6.6
180	52	5.0
overnight	1	

1. Molecular Sieves 10x, 1/8" Pellets

III. Summary

The purpose of the research study was to investigate the feasibility of freeze-dehydrating biological materials by extracting frozen moisture with superdry cryogenic liquids. Extracted moisture was continuously removed by circulating the liquid through a bed of molecular sieves or other suitable dessicant. The data obtained from the experimental work indicates the following:

1. Freeze-dehydration of frozen hydrated gelatin capsules in liquid nitrogen (-196° C) and liquid nitrous oxide (-88.5° C) proceeds too slowly for practical evaluation. Attempts to accelerate the rate of moisture removal by adding Freons to the cryogenic liquids and by applying heat during rehydration were not successful.

2. Freeze-dehydration of frozen hydrated gelatin capsules in ethyl ether at dry ice-alcohol temperature (-78.5°C) achieved 70 to 80% drying in approximately 5 days. Only 14% dehydration was achieved with petroleum ether and 60% with absolute alcohol under comparable conditions of processing. It is of interest that under cryogenic conditions moisture diffuses from frozen capsules into both polar and nonpolar solvents.

3. The presence of a dessicant and agitation of the dehydrating cryogenic solvent were found to significantly improve the rate and extent of drying at dry ice-alcohol temperature (-78.5°C).

4. Critical point dehydration with nitrous oxide in the presence of a dessicant was found to completely dehydrate blood, gelatin, and meat tissue. The presence of a dessicant in the system was determined to be essential for complete dehydration. The dehydrated meat tissues appeared shrunken and did not rehydrate well.

5. A sequential vacuum freeze drying - critical point dehydration process was evaluated for drying meat tissue. Complete drying to a porous product comparable to regular vacuum freeze-dried, products was obtained. With the sequential process, drying from 10% residual moisture to less than 1% moisture was achieved in 5 hrs as compared to 20 hrs by regular vacuum freeze-drying.

6. Histological examination of meat tissue dehydrated by the sequential freeze dry - critical point technique revealed no significant alteration of structure or composition.

7. Significantly lower recovery of viable microorganisms was obtained when dehydrated by the freeze dry - critical point technique. However, the fact that viability retention was obtained under the conditions of elevated temperature and pressure characteristic of critical point dehydration is deemed to be significant.

8. A modified freeze dehydration procedure is recommended for removing moisture from partially vacuum freeze-dried biological materials (10% residual moisture). This entails extracting the residual moisture in a solvent at dry ice-alcohol temperature (-78.5°C) in the presence of a dessicant. Preliminary trials with microorganisms indicated that an innocuous solvent remains to be found as the dehydrating medium. The procedure as such appears to be feasible and would eliminate artifacts and possibly permit higher retention of viability.

IV. Recommendations

1. Studies should be undertaken to develop further the technique of freeze-dehydration in dry cryogenic liquids. Included in the variables to be investigated are: (1) choice of dessicant, (2) sample size, (3) need for agitation of the dehydrating liquid, and (4) toxicity of the liquid.

2. Further consideration should be given to the action of dessicants at cryogenic temperatures with regard to moisture absorption rate, pore size, and efficiency after regeneration at low temperatures.

3. Removing residual moisture from partially freeze-dried biological materials by critical point dehydration or extraction in a dry cryogenic liquid system appears promising. Studies are required to elucidate effects of the dehydration on structural integrity and retention of viability. An investigation of means to accelerate the rate of dehydration is also indicated.

4. A screening study should be performed to endeavor to obtain cryogenic solvents that are innocuous to biological systems.

5. Various low temperatures other than -78.5°C should be investigated for use in the dry cryogenic liquid system. Rates of drying, effects of ice crystallization patterns, structural alterations, and viability retention need to be integrated into the evaluation of the systems.

6. The possible advantages of initial rapid freezing in liquid nitrogen followed by dehydration in a cryogenic liquid at -78.5°C or higher should be investigated particularly with reference to retention of viability.